antibodies bind to an epitope with residues 1-25 of Aβ. Some monoclonal antibodies bind to an epitope within amino acids 1-5, 5-10, 10-15, 15-20, 25-30, 10-20, 20-30, or 10-25 of Aβ. Prophylactic and therapeutic efficacy of antibodies can be tested using the transgenic animal model procedures described in the Examples.

C1 cont

IN THE CLAIMS:

33. A method of preventing or treating a disease characterized by amyloid deposit comprising Aβ peptide, the method comprising administering a polynucleotide encoding at least one antibody chain to the patient in an effective regime whereby the polynucleotide is expressed to produce the antibody chain and the antibody chain reduces levels of Aβ in the brain of the patient.

Sub D6

C2

add D6

REMARKS

Claims 33 and 34 are under consideration. Claim 33 has been amended so that it no longer depends from a canceled base claim. The amendment to claim 33 adds no new matter.

To correct an obvious typographical error, the specification has been amended by replacing the paragraph beginning at line 25 of page 14 with a replacement paragraph. The paragraph was replaced to amend "20, 30" to "20-30."

Priority

Applicant submits herewith a Supplemental Application Data Sheet (ADS). The priority claim made in the Supplemental ADS entitles the instant application to an effective filing date of December 2, 1997.

Information Disclosure Statement

Applicant will submit a Supplemental Information Disclosure Statement, along with all of the references cited therein, for the instant application. This submission will rectify any outstanding issues concerning the IDS in the instant application.

Claim Objections

Claims 33-34 are objected to for depending from a canceled base claim. Claim 33 has been amended to no longer depend from a canceled base claim. Thus, the claim objections have been mooted.

Claim Rejections

Written Description Rejection of Claims 33-34

Claims 33-34 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Office Action alleges the written description requirement is not met because "[t]he specification fails to disclose any nucleic acid or amino acid sequences. In particular, no sequences which specifically encode either the heavy or light chains of an antibody which would specifically bind to an amyloid deposit." (*See* page 4, ¶2 of the Office Action.) Applicant respectfully traverses.

The PTO's Guidelines for application of the written description requirement explicitly recognize that a class of antibodies can be defined in functional terms without providing sequence data. That is a claim to "[a]n isolated antibody capable of binding to antigen X," meets the guidelines notwithstanding lack of any sequence data for the antibody. The functional definition of an antibody is sufficient because of "the routine art-recognized method of making antibodies to fully characterized antigens, the well defined structural characteristics for the five classes of antibody, the functional characteristics of antibody binding, and the fact that the antibody technology is well developed and mature." *See* Example 6 of the Synopsis of Application of Written Description Guidelines.

The cases cited by the Examiner are distinguished from the present facts. The *Vas-Cath* case arose in the typical context of determining new matter. Specifically, the issue was whether drawings of a catheter in a design application provided written description of claims that appeared in a utility application claiming priority to the design application. However, here the Examiner's rejection is applied to originally filed claims and no issue of new matter has been

raised.[1] *Vas-Cath* does not address what written description is required for originally filed claims.

The Fiers, Amgen, and Fiddes cases address written description in situations in which the invention lies in cloning a nucleic acid encoding a particular protein, such as human EPO or FGF for the first time. In circumstances in which the invention lies in cloning a gene, it is perhaps not unreasonable that a newly isolated gene cannot be described without determining its sequence. By contrast, in the present claims, the invention lies not in the de novo isolation of a new gene, such as EPO, but rather in the use of nucleic acids encoding a class of antibodies to a well characterized antigen (Aβ) for a particular purpose (*i.e.*, treating an amyloidogenic disease characterized by deposits of Aβ). In such an invention, it is submitted that written description is provided by the recital of a well characterized antigen (Aβ), the well known conserved features of antibodies, and the mature states of the art as provided in the Guidelines.

For these reasons, withdrawal of the rejection is respectfully requested.

Lack of Enablement Rejection of Claims 33-34

Claims 33-34 are rejected under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement. The rejection is based on several grounds that will be addressed in turn.

First, the Examiner cites Peterson as evidencing difficulties in antibodies folding in bacteria. However, the present claims are directed to expression of antibodies in a patient, not in bacteria. Bacteria do not naturally produce antibodies, so difficulties in folding can be expected from the unnatural environment in which folding occurs in bacteria. These difficulties would not be expected to apply to patients in which immunoglobulins are naturally expressed and properly folded.

Second, the Examiner says the specification does not exemplify particular nucleic acids or expression methods. However, the specification does exemplify several antibodies that bind to Aβ (see Table 13, p. 77). Further, in view of the well-characterized state and commercial availability of Aβ, it would be a routine matter to produce additional antibodies to Aβ (see

[1] The claims are amended in this amendment. However, the rejection as phrased in the ffice action was directed to the original claims.

routine matter to determine the sequence of nucleic acids encoding the antibody chains. Primers for amplifying flanking sequences of antibody variable regions are well known (*see e.g.*, Huse, in Antibody Engineering, (Freeman 1992), Ch. 5), and it is a routine matter to amplify the nucleic acids using such primers, and then sequence the nucleic acids. With respect to means of expression, the Examiner's attention is directed to pp., 25-26 which discusses a number of well known regulatory elements and delivery vectors that can be used.

Third, the Examiner says the claims encompass treatment of any disease associated with amyloid deposit whereas the specification discloses antibodies capable of binding Aβ and which may be useful in decreasing amyloid burden in the brain. In response, the claims have been amended to expedite prosecution to focus on antibodies binding to Aβ as shown in the Examples of the application. However, it is noted the in fact the specification does disclose that the principles exemplified for treatment of diseases characterized by Aβ deposits also apply to other amyloidogenic disease (see p. 23, lines 17-24).

Fourth, the Examiner faults the specification for not showing improvements in clinical and neuropsychological manifestations of Alzheimer's disease as discussed by Morris. However, the specification shows reduction of Aβ depositions in the brains of transgenic mice treated with anti-Aβ antibodies (*see* Example XI); and, a correlation has been shown between a reduction in Aβ deposits and an increase in cognitive performance in transgenic mice. Transgenic mice vaccinated with Aβ showed improved cognitive performance when compared to control transgenic mice, and ultimately performed as well as nontransgeneic mice. The Aβ vaccinated mice also had a partial reduction in amyloid burden at the end of the study. (*See* Morgan *et al.*, *Nature*, 408, pp 982-985; and, Janus *et al.*, *Nature*, 498, pp 979-982, attached hereto.)

Lastly, Applicant respectfully points out that Morris in fact states that diagnoses based on neuropsychological measures "must await confirmation by longitudinal observations and, ultimately by histopathologic examination" (*see* p. 1163, second column). Thus, according to Morris, the histopathologic evidence provided in the present examples is a more definitive indicator of Alzheimer's disease than neuropsychological measures.

For all these reasons, it is submitted that the rejection should be withdrawn.

Rejection of Claims 33-34 Under 35 U.S.C. § 112, Second Paragraph

A. Claims 33-34 are rejected under 35 U.S.C. § 112, second paragraph, as lacking antecedent basis for the limitations "the antibody" and "the patient."

As discussed above, claim 33 has been amended so that it no longer depends from canceled claim 1. Claim 33 as amended provides sufficient antecedent basis for "the antibody" and "the patient" and, thus mooting the rejection under 35 U.S.C. § 112, second paragraph.

B. Claims 33-34 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being incomplete for omitting essential structural cooperative relationships of elements, such as omission amounting to a gap between the necessary structural connections. The alleged structural cooperative relationships are the particular nucleic acids and means of expression.

The issue of omitted essential elements is addressed by MPEP 2172.01, which has two clauses. The first clause states that a "claim which omits matter disclosed to be essential to the invention as described in the specification or other statements of record may be rejected under 35 U.S.C. 112, first paragraph, as not enabling." The second clause states that a "claim which fails to interrelate essential elements of the invention as defined by the applicant(s) in the specification may be rejected under 35 U.S.C. 112, second paragraph."

Here, the Examiner has not indicated any difficulty in understanding the relationship is between existing claim elements or how the alleged omitted elements would clarify this relationship. Rather, the Examiner appears to be requiring that applicants insert new elements into the claim. As such, the rejection has been made under 112, first paragraph rather than second paragraph.

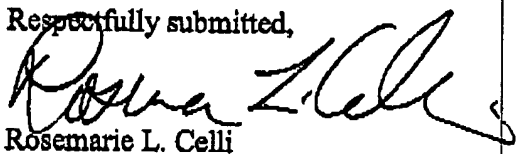
More importantly, in both clauses of MPEP 2172.01 "essential elements" of the invention are those defined as such in the specification or from other statements of record. Here, the specification does not define any specific nucleic acid as being essential. Also, the specification does not define any expression system as being essential but rather describes a number of different expression systems. Because neither a particular nucleic acid or a particular expression system is defined as being essential MPEP 2172.01 does not require that any of these methods, r their component steps be recited in the claims.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-326-2400.

Respectfully submitted,



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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

Please replace the paragraph beginning at line 25 of page 14 with the following replacement paragraph.

Polyclonal sera typically contain mixed populations of antibodies binding to several epitopes along the length of Aβ. Monoclonal antibodies bind to a specific epitope within Aβ that can be a conformational or nonconformational epitope. Some monoclonal antibodies bind to an epitope within residues 1-28 of Aβ (with the first N terminal residue of natural Aβ designated 1). Some monoclonal antibodies bind to an epitope with residues 1-10 of Aβ. Some monoclonal antibodies bind to an epitope with residues 1-16 of Aβ. Some monoclonal antibodies bind to an epitope with residuss 1-25 of Aβ. Some monoclonal antibodies bind to an epitope within amino acids 1-5, 5-10, 10-15, 15-20, 25-30, 10-20, 20-30~~20, 30~~, or 10-25 of Aβ. Prophylactic and therapeutic efficacy of antibodies can be tested using the transgenic animal model procedures described in the Examples.

IN THE CLAIMS:

33. [The method of claim 1,]A method of preventing or treating a disease characterized by amyloid deposit comprising Aβ peptide, the method comprising [wherein the antibody is administered by] administering a polynucleotide encoding at least one antibody chain to the patient in an effective regime[, wherein] whereby the polynucleotide is expressed to produce the antibody chain and the antibody chain reduces levels of Aβ in the brain of [in] the patient.

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This work was supported by grants from the MRC and the Cunningham Trust. We are indebted to Bill Neilon (CJD Surveillance Unit, Edinburgh) and Karen Khan (Elan Pharmaceuticals) for assistance with immunocytochemistry and image analysis.

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Aβ peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease

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Much evidence indicates that abnormal processing and extracellular deposition of amyloid-β peptide (Aβ), a proteolytic derivative of the β-amyloid precursor protein (βAPP), is central to the pathogenesis of Alzheimer's disease (reviewed in ref. 1). In the PDAPP transgenic mouse model of Alzheimer's disease, immunization with Aβ causes a marked reduction in burden of the brain amyloid[2,3]. Evidence that Aβ immunization also reduces cognitive dysfunction in murine models of Alzheimer's disease would support the hypothesis that abnormal Aβ processing is essential to the pathogenesis of Alzheimer's disease, and would encourage the development of other strategies directed at the 'amyloid cascade'. Here we show that Aβ immunization reduces both deposition of cerebral fibrillar Aβ and cognitive dysfunction in the TgCRND8 murine model of Alzheimer's disease without, however, altering total levels of Aβ in the brain. This implies that either a ~50% reduction in dense-cored Aβ plaques is sufficient to affect cognition, or that vaccination may modulate the activity/abundance of a small subpopulation of especially toxic Aβ species.

To explore the behavioural consequences of Aβ immunization, we used the TgCRND8 murine model of Alzheimer's disease that expresses a mutant (K670N/M671L and V717F) human βAPP_{695} transgene under the regulation of the Syrian hamster prion promoter on a C3H/B6 strain background (M.A.C. *et al.*, manuscript in preparation). TgCRND8 mice have spatial learning deficits at 3 months of age that are accompanied by both increasing levels of SDS-soluble Aβ and increasing numbers of Aβ-containing amyloid plaques in the brain. Age- and sex-matched TgCRND8 mice and non-Tg littermates in three cohorts were vaccinated at 6, 8, 12, 16 and 20 weeks with either $A\beta_{42}$ or islet-associated polypeptide (IAPP), which has similar biophysical properties to Aβ but is associated with a non-central nervous system (CNS) amyloidosis. Both immunogens were in β-pleated-sheet conformation at the

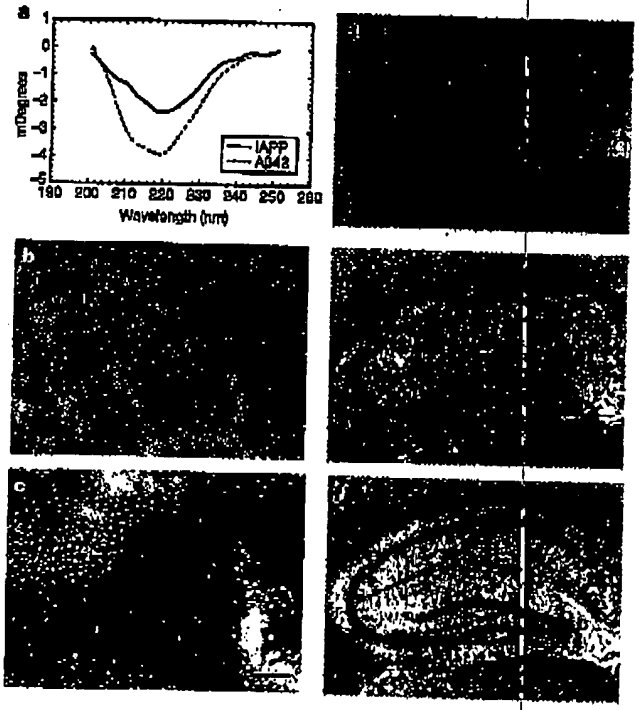


Figure 1 Aβ and IAPP peptide immunogens were predominantly β-structured and induced antibodies recognizing fibrillar Aβ deposits. **a**, Circular dichroism spectra of $A\beta_{42}$ (dotted line) and IAPP (solid line) before immunization are predominantly β-structured. *m*Degrees, millidegrees. **b**, Negative-stain electron microscopy (scale 150 nm) of $A\beta_{42}$ peptide immunogen showed varying length fibres; **c**, IAPP peptide immunogen showed short laterally aggregated fibres. **d**, **f**, Serum (1:1,000 dilution) from non-immunized TgCRND8 mice (**d**) and from 23-week old IAPP-immunized mice (**f**) did not recognize the Aβ plaques in adjacent, non-formic-acid treated sections from a non-immunized TgCRND8 mouse with abundant Aβ-positive plaques. **e**, Sera from $A\beta_{42}$-immunized mice strongly labelled dense-cored amyloid plaques but not diffuse Aβ deposits (which are profusely present in these animals and were labelled with other anti-Aβ antibodies such as 4G8; ref. 28).

time of injection (Fig. 1) and induced detectable antibody titres in all mice by 13 weeks of age (as measured by enzyme-linked immunoabsorbent assay (ELISA) using fibrillar forms of the respective immunogen). These titres increased by a further ~2–3-fold at 23 weeks ($A\beta_{42}$ titres in $A\beta_{42}$-immunized mice (mean ± s.e.m.): 1:3,640 ± 470 at 13 weeks; 1:7,500 ± 1,712 at 23 weeks; IAPP titres in IAPP-immunized mice: 1:3,839 ± 1,167 at 13 weeks; 1:11,500 ± 3661 at 23 weeks). The sera from Aβ-immunized mice intensely decorated extracellular, dense-cored plaque deposits when applied as an immunohistochemical reagent to sections of brain from TgCRND8 mice containing abundant amyloid plaques (which predominantly display Aβ in a β-sheet conformation) or to formic-acid-treated sections (which also display additional non-β-sheet Aβ-epitopes) (Fig. 1). However, these sera reacted only very weakly with diffuse, non-fibrillar Aβ deposits, which can be readily detected in these tissues by anti-Aβ monoclonal antibodies such as 4G8 (data not shown). The Aβ-immune sera did not stain normal neurons, indicating limited crossreactivity with βAPP holoprotein. In contrast, sera from non-immunized or IAPP-immunized mice did not stain any structures. Together, these data indicate that in this strain of mice immunization with $A\beta_{42}$ protofibrillar assemblies induced antibodies directed primarily towards Aβ in a β-sheet conformation.

The mice were tested longitudinally in a reference memory version of the Morris water maze test at 11, 15, 19 and 23 weeks (Fig. 2). At each age of testing, the hidden platform was placed in a different quadrant of the pool. These data were analysed for the entire test period using a mixed model analysis of variance (ANOVA), with immunogen ($A\beta_{42}$ versus IAPP) and genotype (TgCRND8 versus non-Tg) as a between-subject factor, and age-of-testing (11, 15, 19 and 23 weeks) as a within-subject factor. This longitudinal design and mode of analysis, which simulates longitudinal human clinical trials[4], revealed that $A\beta_{42}$-immunized TgCRND8 mice performed significantly better than IAPP-immunized TgCRND8 mice ($P < 0.05$), with 31% of the performance variance being due to the effects of the immunogen. However, the improvement was partial—the $A\beta_{42}$-immunized TgCRND8 mice did not perform as well as their non-Tg littermates ($P < 0.01$). Because the experimental design involved testing of naive mice at 11 weeks of age, followed by a series of reversal tests at 15, 19 and 23 weeks, an additional analysis carried out on the reversal tests confirmed improved performance for the Aβ-immunized Tg mice ($P < 0.02$) during this phase of testing as well. The overall conclusion that $A\beta_{42}$ immunization ameliorates the cognitive deficit of TgCRND8 mice was robust, regardless of whether the analysis assessed latency to reach the hidden platform or swim path length (a measure that is less sensitive to swim speed and floating[5]).

The improved performance of $A\beta_{42}$-immunized TgCRND8 mice was not due to a nonspecific effect of immunization or to an effect on other behavioural, motor, or perceptual systems. Control studies

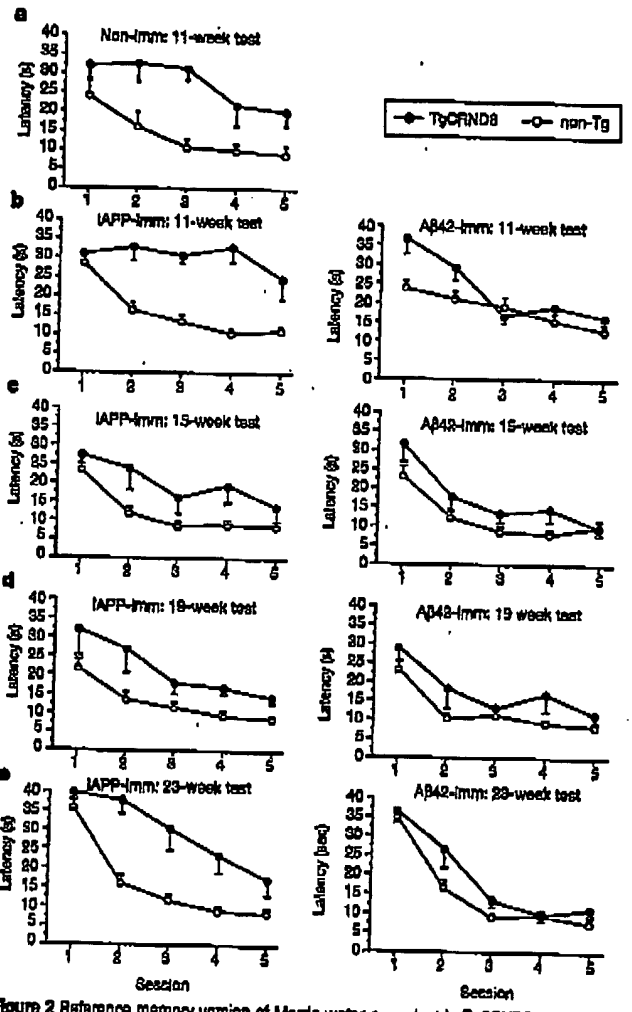
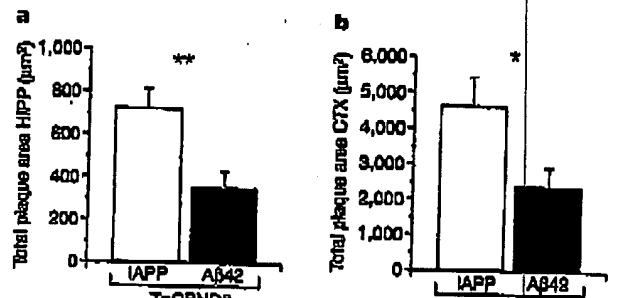


Figure 2 Reference memory version of Morris water maze test in TgCRND8 mice. At 11 weeks of age, non-immunized TgCRND8 mice ($n = 5$) show cognitive impairment relative to non-Tg controls ($n = 8$) (a), which is similar to that of IAPP-immunized TgCRND8 mice ($n = 12$) (b, left), whereas the performance of $A\beta_{42}$-immunized TgCRND8 mice ($n = 9$) (b, right) approaches that of non-Tg littermates ($n = 19$). At 15 (c) and 19 (d) weeks of age, the IAPP-immunized TgCRND8 mice ($n = 6$) (left) were impaired compared with non-Tg littermates ($P < 0.01$, $\omega^2 \approx 36\%$; $n \approx 18$), but were not significantly different from the $A\beta_{42}$-immunized TgCRND8 mice ($n = 6$) (right). At 23 weeks of age, the IAPP-immunized TgCRND8 mice ($n = 6$) (e, left) were significantly impaired relative to both non-Tg littermates ($n = 15$; $P < 0.001$, $\omega^2 = 65\%$) and $A\beta_{42}$-immunized TgCRND8 mice ($n = 6$; $P < 0.01$) (e, right). Vertical bars represent s.e.m. See text and Supplementary Information for statistical analyses.



IAPP-immunized TgCRND8 Aβ42-immunized TgCRND8

Figure 3 At 25 weeks of age, in TgCRND8 mice immunized with $A\beta_{42}$ peptides, dense-cored Aβ plaque burden is reduced in the hippocampus (HIPP) (a) and in the cerebral cortex (CTX) (b). The $A\beta_{42}$-immunized TgCRND8 mice had 50% fewer plaques than IAPP-immunized TgCRND8 controls (71.4 ± 10.8 per area counted versus 119.7 ± 14.6 in the cortex, $P < 0.05$; and 11.6 ± 1.6 per area counted versus 20.9 ± 1.7 in hippocampus, $P < 0.01$). Representative pictures of the distribution of Aβ plaques labelled by the Dako 6F/3D anti-Aβ monoclonal antibody in hippocampus of IAPP- and $A\beta_{42}$-immunized TgCRND8 mice (c and d, respectively). Vertical bars represent s.e.m. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$. Scale bars, 100 μm.

Supplementary Information for more details) in serum samples (200 µl of blood) collected at 19 and 25 weeks.

Behavioural tests and data analysis

The water maze apparatus, mouse handling and general testing procedures have been described[23]. Before the first spatial learning test at 11 weeks, all mice underwent non-spatial pre-training (NSP) to assess swimming abilities and to accustom mice to the test[24,25] (see Supplementary Information). Two days after the NSP phase, all mice underwent a reference memory training with a hidden platform placed in the centre of one quadrant of the pool for 5 days, with four trials per day. After the last trial of day 5, the platform was removed from the pool and each mouse received one 60-s swim probe trial. Escape latency (s), length of swim path (cm), swim speed (cm s^{-1}), % of floating (speed less than 5 cm s^{-1}), % of time in outer zone (near the pool wall), and % of time and path in each quadrant of the pool were recorded using an on-line HVS image video tracking system[23] (see Supplementary Information).

For the probe trials, an annulus-crossing index was calculated that represents the number of passes over the platform site, minus the mean of passes over alternative sites in other quadrants. The index expresses the spatial place preference and controls for alternative search strategies without place preferences, such as circular search paths[26,27]. All mice were re-tested at 15, 19 and 23 weeks of age, one week before the next immunization. At each re-testing, the platform was placed in the centre of a different, semi-randomly chosen pool quadrant for all five sessions of training. At the end of the experiment, all mice were given a cue (visual platform) learning test. This was followed by the open-field test to investigate spontaneous locomotor exploration. Behavioural data was analyzed using a mixed model of factorial ANOVA. Degrees of freedom were adjusted by Greenhouse–Geisser epsilon correction for heterogeneity of variance. A Bonferroni Inequality correction was applied for multiple comparisons. Omega squared (ω^2) was used as a measure of effect size caused by different factors.

Analysis of βAPP and amyloid burden in brain

Three 5-µm sections at 25-µm intervals from one cerebral hemisphere were immunostained with Dako 6F/3D anti-Aβ monoclonal antibody to residues 8–17 (which is primarily reactive against dense-cored plaques) with 4GB (ref. 28), or with sera from immunized mice, and counterstained with haematoxylin and resin mounted as described (M.A.C. *et al.*, manuscript in preparation). For some samples the formic-acid treatment step was omitted. End products were visualized with diaminobenzidine. Amyloid plaque burden was assessed using Leco IA-3001 image analysis software interfaced with a Leica microscope and a Hitachi KP-M1U CCD video camera. The quantitative analysis was performed at of ×25 magnification, and the image frame and guard size was set to 0,0,639,479 (307,200 µm^2) for each slide. The brain area (cortex or hippocampus) was outlined using the edit plane function, and the area and number of plaques in the outlined structure were recorded. Data were pooled for all three sections.

Cerebral Aβ levels were assayed from formic-acid-extracted[29], hemi-brain sucrose homogenates using an ELISA method (see Supplementary Information) in which Aβ was trapped with either monoclonal antibody to $Aβ_{40}$ (JRF/cAb40/10) or $Aβ_{42}$ (JRF/cAb42/26) and then detected with horseradish peroxidase (HRP)-conjugated JRF/Abtot/17. The dilution of JRF/Abtot/17 and samples were optimized to detect Aβ in the range of 50 to 800 fmol ml^{-1}. ELISA signals are reported as the mean ± s.e.m. of four replica wells in fmol Aβ per mg total protein (determined with the BioRad DC protein assay), based on standard curves using synthetic $Aβ_{1-40}$ and $Aβ_{1-42}$ peptide standards (American Peptide Co. Sunnyvale, CA). Cerebral βAPPs levels were analyzed in supernatant of brain as described[30].

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Aβ peptide vaccination prevents memory loss in an animal model of Alzheimer's disease

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Vaccinations with amyloid-β peptide (Aβ) can dramatically reduce amyloid deposition in a transgenic mouse model of Alzheimer's disease[1]. To determine if the vaccinations had deleterious or beneficial functional consequences, we tested eight months of Aβ vaccination in a different transgenic model for

Supplementary Information for more details) in serum samples (200 µl of blood) collected at 13 and 25 weeks.

Behavioural tests and data analysis

The water maze apparatus, mouse handling and general testing procedures have been described[25]. Before the first spatial learning test at 11 weeks, all mice underwent non-spatial pre-training (NSP) to assess swimming abilities and to accustom mice to the test[24,25] (see Supplementary Information). Two days after the NSP phase, all mice underwent a reference memory training with a hidden platform placed in the centre of one quadrant of the pool for 5 days, with four trials per day. After the last trial of day 5, the platform was removed from the pool and each mouse received one 60-s swim probe trial. Escape latency (s), length of swim path (cm), swim speed (cm s^{-1}), % of floating (speed less then 5 cm s^{-1}), % of time in outer zone (near the pool wall), and % of time and path in each quadrant of the pool were recorded using an on-line HVS Image video tracking system[25] (see Supplementary Information).

For the probe trials, an annulus-crossing index was calculated that represents the number of passes over the platform site, minus the mean of passes over alternative sites in other quadrants. The index expresses the spatial place preference and controls for alternative search strategies without place preferences, such as circular search paths[24,25]. All mice were re-tested at 15, 19 and 23 weeks of age, one week before the next immunization. At each re-testing, the platform was placed in the centre of a different, semi-randomly chosen pool quadrant for all five sessions of training. At the end of the experiment, all mice were given a cue (visual platform) learning test. This was followed by the open-field test to investigate spontaneous locomotor exploration. Behavioural data was analysed using a mixed model of factorial ANOVA. Degrees of freedom were adjusted by Greenhouse–Geisser epsilon correction for heterogeneity of variance. A Bonferroni inequality correction was applied for multiple comparisons. Omega squared (ω^2) was used as a measure of effect size caused by different factors.

Analysis of βAPP and amyloid burden in brain

Three 5-µm sections at 25-µm intervals from one cerebral hemisphere were immunostained with Dako 6F/3D anti-Aβ monoclonal antibody to residues 8–17 (which is primarily reactive against dense-cored plaques) with 4G8 (ref. 28), or with sera from immunized mice, and counterstained with haematoxylin and resin mounted as described (M.A.C. *et al.*, manuscript in preparation). For some samples the formic-acid treatment step was omitted. End products were visualized with diaminobenzidine. Amyloid plaque burden was assessed using Leco IA-3001 image analysis software interfaced with a Leica microscope and a Hitachi KP-M1U CCD video camera. The quantitative analysis was performed at of × 25 magnification, and the image frame and guard size was set to 0,0,639,479 (307,200 µm²) for each slide. The brain area (cortex or hippocampus) was outlined using the edit plane function, and the area and number of plaques in the outlined structure were recorded. Data were pooled for all three sections.

Cerebral Aβ levels were assayed from formic-acid-extracted[29], hemi-brain sucrose homogenates using an ELISA method (see Supplementary Information) in which Aβ was trapped with either monoclonal antibody to $Aβ_{40}$ (JRF/cAb40/10) or $Aβ_{42}$ (JRF/cAb42/26) and then detected with horseradish peroxidase (HRP)-conjugated JRF/Abtot/17. The dilution of JRF/Abtot/17 and samples were optimized to detect Aβ in the range of 50 to 800 fmol ml^{-1}. ELISA signals are reported as the mean ± s.e.m. of four replica wells in fmol Aβ per mg total protein (determined with the BioRad DC protein assay), based on standard curves using synthetic $Aβ_{1-40}$ and $Aβ_{1-42}$ peptide standards (American Peptide Co. Sunnyvale, CA). Cerebral βAPP$_S$ levels were analysed in supernatant of brain as described[30].

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Vaccinations with amyloid-β peptide (Aβ) can dramatically reduce amyloid deposition in a transgenic mouse model of Alzheimer's disease[1]. To determine if the vaccinations had deleterious or beneficial functional consequences, we tested eight months of Aβ vaccination in a different transgenic model for

Alzheimer's disease in which mice develop learning deficits as amyloid accumulates[1,2]. Here we show that vaccination with Aβ protects transgenic mice from the learning and age-related memory deficits that normally occur in this mouse model for Alzheimer's disease. During testing for potential deleterious effects of the vaccine, all mice performed superbly on the radial-arm water-maze test of working memory. Later, at an age when untreated transgenic mice show memory deficits, the Aβ-vaccinated transgenic mice showed cognitive performance superior to that of the control transgenic mice and, ultimately, performed as well as nontransgenic mice. The Aβ-vaccinated mice also had a partial reduction in amyloid burden at the end of the study. This therapeutic approach may thus prevent and, possibly, treat Alzheimer's dementia.

The accumulation of fibrils formed from the Aβ peptide into amyloid plaques is a defining characteristic of Alzheimer's disease (AD). The Aβ vaccination protocol described in ref. 1 reduced Aβ deposits, which suggested that this approach might benefit AD patients. However, the functional consequences of such vaccinations might be deleterious. For example, plaque-associated inflammation promoted by the immunization could interfere with normal brain functioning, and/or lead to degenerative changes in the brain[4–6]. We used a novel working-memory task that combines elements of a radial-arm maze and a water maze. This radial-arm water maze is remarkably robust at detecting learning/memory deficits that develop in AD transgenic mice[2] and more efficient in sample size requirements than other memory tasks typically used for rodents[3].

To test the possibility that vaccinations might cause premature memory deficits in AD transgenic mice, we assessed learning/memory performance in the mice at 11.5 months of age after five inoculations with Aβ or the control vaccine, keyhole limpet haemocyanin (KLH). All mice showed strong learning and memory capacity, irrespective of treatment or transgene status (Fig. 1a). All groups averaged three to four errors on the first trial as they sought out the new platform location for that day, but averaged less than one error by trials 4 or 5, demonstrating intact working memory for platform location between trials and during the 30-min delay before trial 5. This strong performance by Aβ-vaccinated mice indicates that any inflammatory responses caused by the vaccine were not deleterious behaviourally.

Monthly inoculations were continued until the mice were 15.5 months, when these mice were tested again in the radial-arm water maze. At 15.5 months the KLH-vaccinated transgenic mice failed to demonstrate learning or memory of the platform location; their performance on all trials was the same (Fig. 1b). This is identical to the performance of other untreated transgenic mice that had been previously tested in this learning task at this age (Fig. 1c; ref. 3). In contrast, the Aβ-inoculated transgenic mice, although slower to learn platform location than nontransgenic mice on trial 3 (Fischer's least

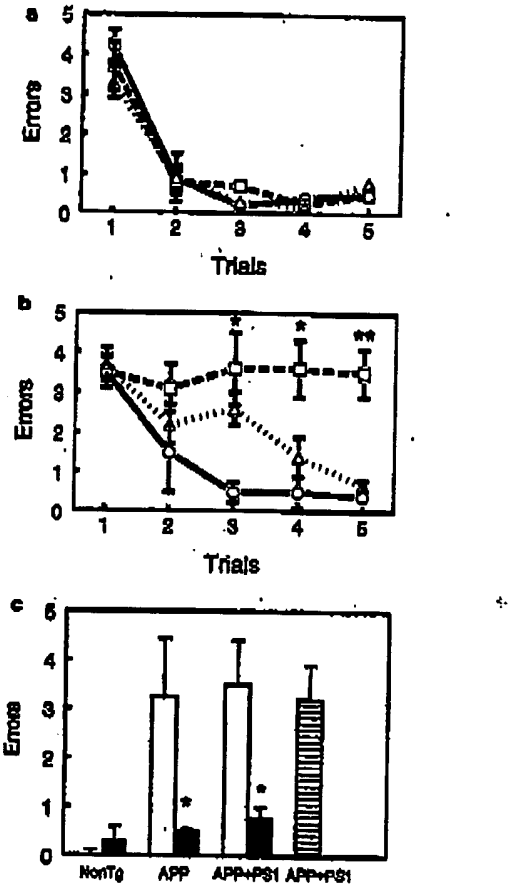

Figure 1 Radial-arm water-maze performance in vaccinated transgenic and nontransgenic mice. a, Nontransgenic mice (circles, solid lines), transgenic mice vaccinated with KLH (squares, dashed lines), and transgenic mice vaccinated with Aβ (triangles, dotted lines) were tested in the radial-arm water maze at 11.5 months of age (after five inoculations). All groups learned (trial 4) and remembered (trial 5) the platform location at this time point. In the same mice at 15.5 months of age (nine inoculations; b), the transgenic mice vaccinated with Aβ continued to show learning and memory of the platform location, whereas the transgenic mice vaccinated with KLH failed to show learning and memory for platform location on either trials 4 or 5 (*$P < 0.05$, **$P < 0.01$; KLH significantly different from other two groups by LSD post hoc analysis after MANOVA). This benefit of Aβ vaccination was found in both the APP-only and APP+PS1 transgenic mice (c), with significantly fewer errors on trial 5 in the Aβ-vaccinated groups (solid bars) than in the KLH-vaccinated group (open bars) of both genotypes (*$P < 0.03$). Included for comparison is the trial 5 performance of another group (hatched bars) of untreated 15–16-month-old transgenic mice that were tested separately, and are reported on fully elsewhere[3].

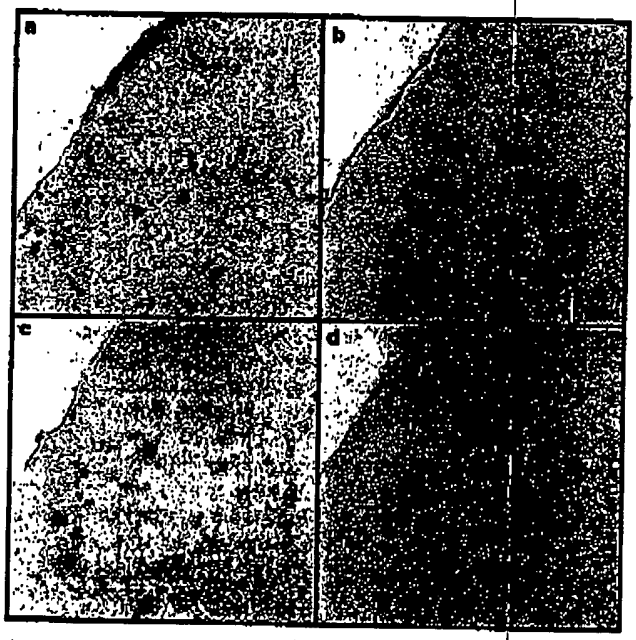

Figure 2 Amyloid pathology in transgenic mice vaccinated with KLH or Aβ. Immunohistochemistry for Aβ in frontal cortex is shown in (KLH-vaccinated) (a) and (Aβ-vaccinated) (b) in transgenic mice with values similar to the means shown in Fig. 3c. Congo-red staining is shown in (KLH) (c) and (Aβ) (d) in mice with values corresponding to the means in Fig. 3b. Horizontal sections are oriented with the corpus callosum in the lower right corner and anterior to the top. Scale bar, 500 μm.

significant difference (LSD), $P < 0.02$), were nearly flawless by trial 5, and performed significantly better than the KLH-vaccinated transgenic mice on both trials 4 and 5 (multiple analysis of variance, MANOVA: $F_{(2,15)} = 5.83$, $P < 0.02$ and $F_{(2,15)} = 12.16$, $P < 0.001$, respectively; KLH transgenic group different from both other groups by Fischer's LSD post hoc comparisons, $P < 0.05$ n trial 4 and $P < 0.01$ on trial 5). Our individual evaluation of the performance of the two transgenic genotypes made it clear that both APP-only and APP+PS1 transgenic mice benefited from the Aβ vaccinations (Fig. 1c).

Serological analysis indicated that mice injected with Aβ developed antibodies against the Aβ peptide. Very high titres were found in both transgenic and nontransgenic mice immunized with Aβ (IC_{50} = 27,000 ± 5,000 and 48,000 ± 18,000, respectively; not significant). There was no anti-Aβ activity in the KLH-immunized transgenic mice, untreated transgenic mice, nor nontransgenic mice at final dilutions of serum down to 1:16, indicating that transgenic mice did not spontaneously generate an antibody reaction to Aβ.

Immunization with Aβ caused a modest reduction in Aβ deposits in the frontal cortex, with a significant reduction in the Congo-red-stained area of APP+PS1 mice, and a significant reduction in the Aβ-immunostained area of APP mice (Fig. 2 and Fig. 3). Reductions of a similar extent were found in hippocampus. We also quantified immunostaining using Aβ40- and Aβ42-specific antisera, both of which exhibited the same modest reductions found in total Aβ immunostaining. We suspect that, with a larger sample size, statistically significant partial reductions would be found in all these measures consistent with other recent reports[9–11]. In general, the percentage reduction in Aβ deposition was greater in the APP mice than the APP+PS1 mice. The absolute reductions were greater, however, in the doubly transgenic animals. The APP+PS1 mice already had substantial Aβ deposits by the time vaccinations were initiated[12]. Further studies will test whether beginning vaccinations at an earlier age, or combining vaccination with other Aβ-lowering treatments, will result in more complete protection from Aβ deposition, and improve the cognitive performance of 15-months-old transgenic mice even further.

Our most important finding here is that Aβ vaccination protects transgenic mice from developing memory deficits compared with KLH-immunized (control) transgenic mice. But how important is the learning paradigm in discerning these differences. We have found that in using the reference-memory version of the water maze, mice of this age (15.5 months) have deficits in escape latency, but not retention on the probe trial[3]. Thus, the more demanding working-memory version of the water-maze task may be essential to detect such differences. Similarly, a spatial task would require intact function of hippocampal and, to a lesser extent, cortical structures, the locations where plaques accumulate earliest and to the greatest extent in these mice[12–14].

This vaccination-associated protection from memory impairment occurs in the presence of reduced, but still substantial Aβ deposits. The mechanism by which immunization with Aβ blocks learning and memory deficits is not understood. One possibility is that the antibodies neutralize Aβ in some restricted compartment or deplete a non-deposited form of Aβ (for example, a soluble form) that is responsible for the memory loss observed. Recently, soluble Aβ has been proposed as the cause of synapse loss in APP transgenic mice, as some transgenic lines develop reductions in synaptophysin immunoreactivity in dentate gyrus without developing Aβ deposits[15]. A second possibility is that microglia activated by the inoculations[1] can clear the deposited Aβ, thereby permitting normal cognitive function. This is not easily reconciled with the relatively modest Aβ clearance detected, although exhaustive regional analyses have yet to be completed. Perhaps even mice that have already developed extensive brain pathology and memory deficits can benefit from vaccinations given later in life. In view of the absence of adverse effects on behaviour and brain functioning, and the protection of memory functions by the Aβ vaccines, we strongly recommend testing of this and related approaches for the treatment and prevention of Alzheimer's disease. □

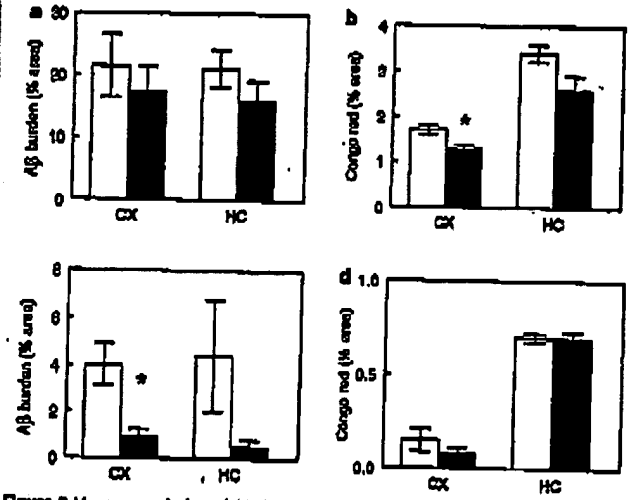

Figure 2 Measurement of amyloid histopathology after Aβ peptide immunization. a, b, Results for the APP+PS1 mice; c, d, results for APP-only transgenic mice. A significant reduction in Congo-red staining in frontal cortex was found in APP+PS1 mice vaccinated with Aβ ($n = 4$) compared to in APP+PS1 mice vaccinated with KLH ($n = 5$; b). There was a significant reduction in Aβ immunostaining in APP-only transgenic mice vaccinated with Aβ ($n = 3$) compared to in KLH-vaccinated APP mice ($n = 2$; c). *, $P < 0.05$; **, $P < 0.01$ by t-test. CX, frontal cortex; HC, hippocampus.

Methods

Vaccination protocols

Mice were obtained by breeding Tg 2576 APP transgenic mice[16] with PS1 line 5.1 transgenic mice[17], resulting in nontransgenic, APP, APP+PS1 and PS1 transgenic mice as described by us previously[12,13]. Human Aβ1–42 peptide (Bachem) was suspended in pyrogen-free Type I water at 2.2 mg ml^{-1} then mixed with 10 × PBS to yield 1 × PBS and incubated overnight at 37 °C. Control mice were injected with KLH that was prepared in the same manner. The antigen suspension was mixed 1:1 with Freund's complete adjuvant and 100 μg Aβ injected subcutaneously by an experimenter who had no role in the behavioural testing. A boost of the same material (prepared freshly) was made in incomplete Freund's at two weeks and injected once monthly for the next three months. Subsequent monthly boosts were made in mineral oil. Mice were vaccinated, beginning at 7.5 months of age. The sample size of each group was: 6 (3 female/3 male) nontransgenic mice vaccinated with Aβ or KLH; 7 (4 female/3 male) transgenic mice vaccinated with Aβ; 7 (4 female/3 male) transgenic mice vaccinated with KLH. The first post-vaccination behavioural testing period was started 5 days after the fifth vaccination at 11.5 months of age. The second behavioural testing period was started at 15.5 months of age, one month after the ninth vaccination. Mice were killed at 16 months of age. We note that transgenic and nontransgenic mice were also tested for performance in the radial-arm water maze at 6 months of age (before vaccination) and all mice performed well.

Radial-arm water maze testing

Experimenters were unaware of the experimental conditions of the mice at the time of testing. The maze consisted of a circular pool 1 m in diameter with six swim alleys (arms) 19 cm wide that radiated out from an open central area (40 cm in diameter), with a submerged escape platform located at the end of one of the arms[3,11]. Spatial cues were present on the walls and ceiling of the testing room. The escape platform was placed in a different arm each day, forcing mice to use working memory to solve the task. Each day, mice were given the opportunity to learn the location of the submerged platform during four consecutive acquisition trials followed 30 min later by a retention trial (trial 5). On each trial, the mouse was started in one arm not containing the platform and allowed to swim for up to one minute to find the escape platform. Upon entering (all four paws within the swim alley) an incorrect arm or failing to select an arm after 20 s, the mouse was gently pulled back to the start arm for that trial and charged an error. All mice spent 30 s on the platform following each trial before beginning the next trial. On subsequent trials that day, the start arm was varied, so the mouse could not simply learn the motor rule 'second arm to the left', but must learn the spatial location of the platform that day. After the fourth trial was completed, the mice were placed in their home cage for 30 min, then returned to the maze and administered the retention trial. The platform was located in the same arm on each trial within a day, and was in a different arm across days. Over 1–2 weeks of

training, control groups gradually improved performance as they learned the procedural aspects of the task, reaching an asymptotic level of 0.5–1 errors on trials 4 and 5. In the experiments presented here mice were trained until the nontransgenic mice reached asymptotic performance: 9 days at 11.5 months or 11 days at 15.5 months. The scores for each mouse on the last two days of testing were averaged and used for statistical analysis. Sensorimotor tests identified no differences among these groups in open field behaviour or string agility testing. As in earlier work, all transgenic mice were impaired on the balance beam, a deficit observed as early as six months of age[3], but this deficit was not modified by Aβ vaccination.

ELISA analysis for serum antibodies

Ninety-six-well Immulon 4HBX (Dynex) micro plates were coated with the Aβ1–42 protein (250 ng per well) for 1 h at 37 °C. They were washed four times with 0.45% NaCl + 0.05% Tween-20 (washing buffer, WB). The plates were blocked with 5% non-fat dry milk (NFDM) in PBS overnight at 4 °C and washed the following day. Mouse serum was prepared in PBS at an initial dilution of 1:16 and subsequent twofold dilutions were made. All samples were run in duplicate and incubated at 37 °C for 1 h followed by washing 10 times in WB. Plates were blocked a second time with 5% NFDM in PBS for 30 min at 37 °C followed by washing five times before the addition of an anti-mouse IgG HRP-conjugate. The secondary antibody was diluted 1:5,000 in PBS and incubated for 1 h at 37 °C. Plates were then washed 10 times in WB and developed with 3,3,5,5-tetramethylbenzidine substrate (Sigma) in perborate buffer (Sigma). The reaction was stopped with 2 M sulphuric acid. Plates were read spectrophotometrically at 450 nm. The anti-Aβ1–42 antibody titre was defined as the reciprocal of the dilution of antisera that produced 50% of the maximum signal detected for that sample.

Histopathology

Mice were overdosed with pentobarbital, perfused with saline and their brains removed. One hemisphere was immersion-fixed in fresh, buffered paraformaldehyde for 24 h. Frozen sections were stained for Aβ peptides by immunohistochemistry[18,19] or for Congo red. The area of frontal cortex occupied by stain was measured with a Videometric V150 image analysis system (Oncor) on a Nikon Microphot FX microscope. Stained regions were measured using HSI segmentation by an experimenter unaware of the subject condition. Both stain intensity and area were measured, although only areas are reported here as this is the convention for Aβ deposits ('amyloid burden'). The results were not qualitatively different when evaluating area, stain intensity or their product (total immunoreactivity[18]). Data were collected from equally spaced horizontal sections for both frontal cortex (anterior to the corpus callosum; 12 per mouse) and hippocampus (10 per mouse).

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Induction of vanilloid receptor channel activity by protein kinase C

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Capsaicin or vanilloid receptors (VRs) participate in the sensation of thermal and inflammatory pain[1–3]. The cloned (VR1) and native VRs are non-selective cation channels directly activated by harmful heat, extracellular protons and vanilloid compounds[4–6]. However, considerable attention has been focused on identifying other signalling pathways in VR activation; it is known that VR1 is also expressed in non-sensory tissue[1,7] and may mediate inflammatory rather than acute thermal pain[3]. Here we show that activation of protein kinase C (PKC) induces VR1 channel activity at room temperature in the absence of any other agonist. We also observed this effect in native VRs from sensory neurons, and phorbol esters induced a vanilloid-sensitive Ca^{2+} rise in these cells. Moreover, the pro-inflammatory peptide, bradykinin, and the putative endogenous ligand, anandamide, respectively induced and enhanced VR activity, in a PKC-dependent manner. These results suggest that PKC may link a range of stimuli to the activation of VRs.

PKC is a prominent participant in pain signalling. Targeted deletion of PKC-ε in mice[10] markedly attenuates thermal- and acid-induced hyperalgesia. In turn, activation of PKC-ε potentiates heat-evoked currents in sensory neurons[11,12]. Further, the algesic peptide, bradykinin, potentiates heat responses[11,12], induces depolarization[13–16], and evokes secretion[17–19] from vanilloid-sensitive neurons in a PKC-dependent manner. However, the molecular targets for these effects have not yet been clearly identified. We therefore investigated whether these actions of PKC are mediated by VRs. Rat VR1 was expressed in *Xenopus laevis* oocytes and studied using a two-electrode voltage clamp technique. Treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to activate endogenous PKC increased the amplitude of currents evoked by capsaicin (Fig. 1a, c), anandamide (Fig. 1b, c) and protons (extracellular pH 5; data not shown). In addition, TPA by itself produced a slowly developing current (Fig. 1a, b) that was not observed in uninjected oocytes ($n = 5$) or oocytes expressing the NMDA (*N*-methyl D-aspartate) receptor ($n = 8$). These actions were probably mediated by PKC because no responses were elicited by the inactive TPA analogue, 4α-phorbol ($n = 4$), and responses to TPA were inhibited by the selective PKC inhibitor[20], bisindolylmaleimide (BIM, 200 nM, Fig. 1c).

Next, we examined whether the current induced by TPA alone was mediated by VR1. In these experiments VR1-expressing oocytes were treated separately with either TPA or capsaicin, to avoid cross-sensitization. Figure 1d shows the response of a TPA-treated oocyte to a series of depolarizing pulses from −80 mV to +80 mV. Outwardly rectified currents were evoked that were similar to those